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(54) Flow-through electrochemical immunoassay biosensor

(57) The sensor comprises a solid phase immunoassay system 4 close to a porous working electrode 5, a counter electrode 6 and means to produce a flow of fluid through the sensor. The solid phase is preferably at least one porous membrane and the fluid flow may be produced by a pump or by a wicking material. The porous working electrode may be made of graphite, carbon paper or may be a printed electrode on porous material. Detection of TSH (thyroid stimulating hormone) and thyroxin is described.

Figure 1

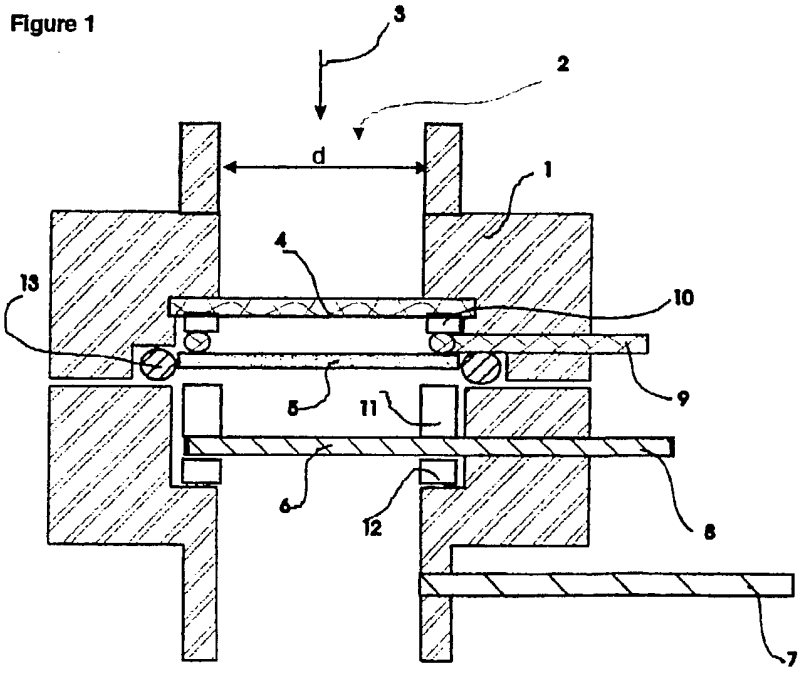

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Figure 1

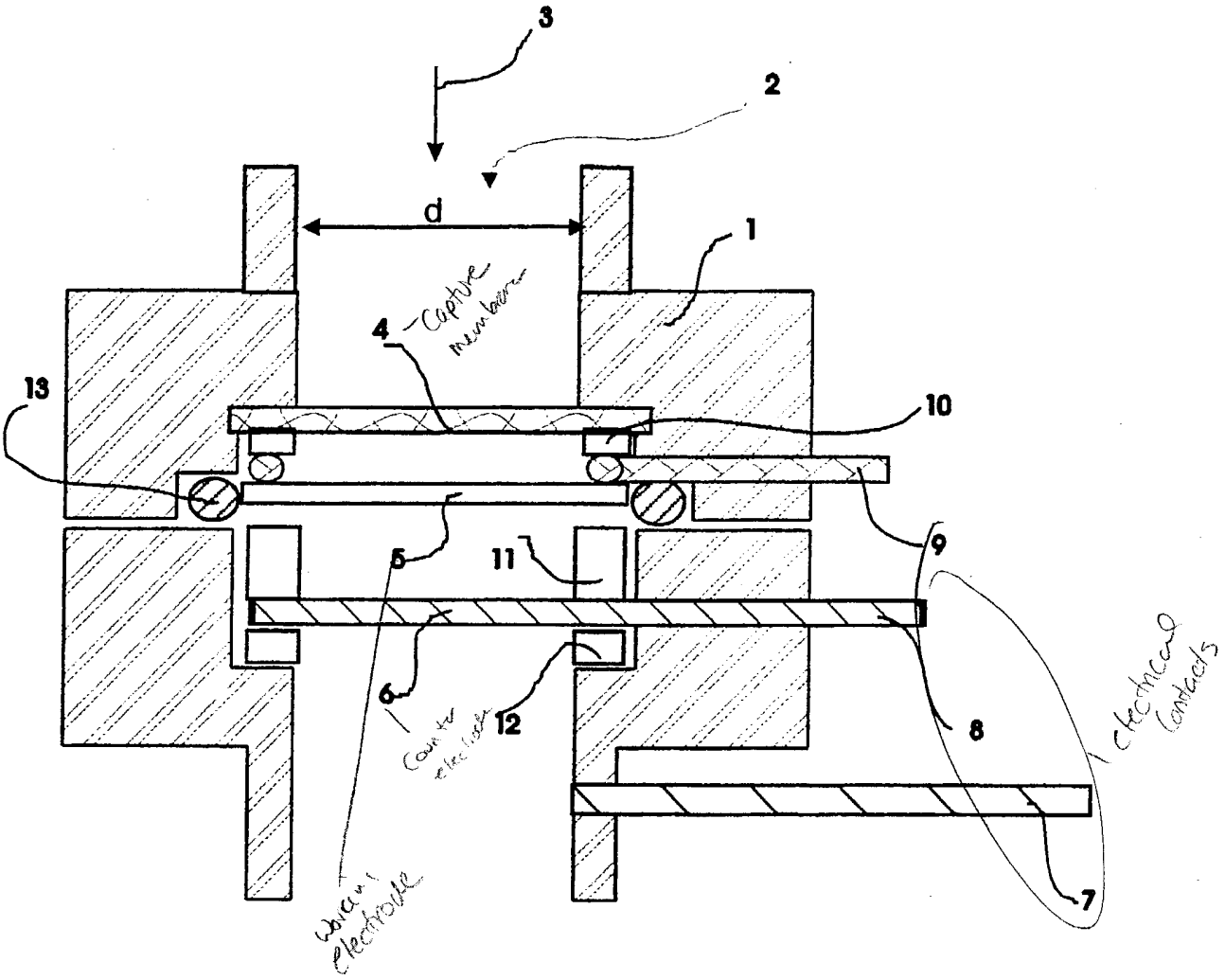

3
2
d
1
4
Capture membrane
13
10
9
5
11
6
12
8
Electrical Contacts
7

Figure 2

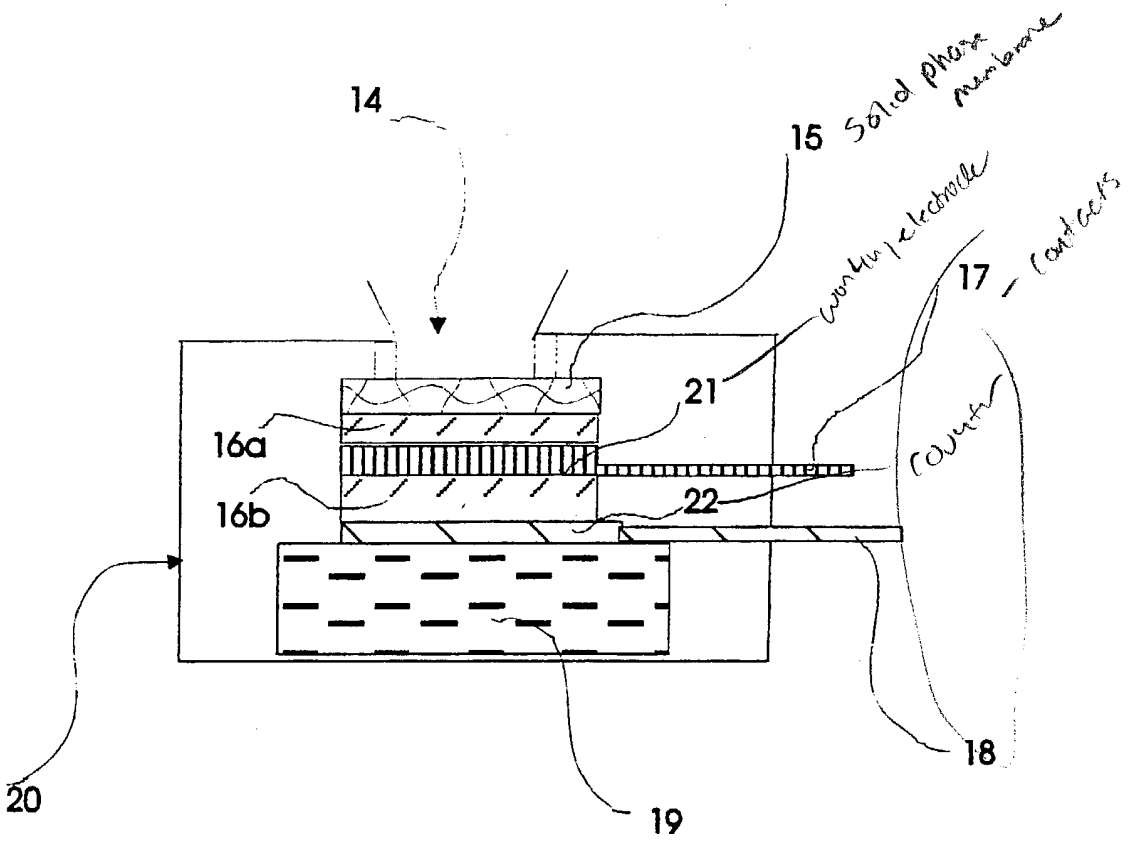
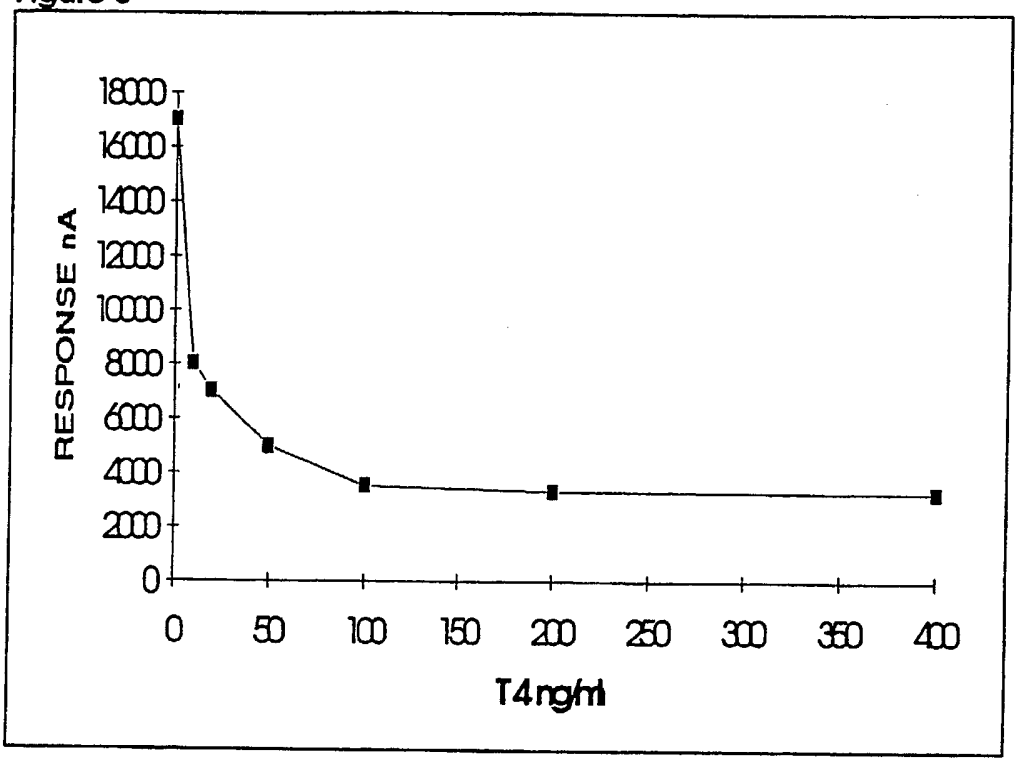

14
15
Solid phase membrane
Working electrode
17 - contacts
21
16a
16b
22
counter
18
19
20

Figure 3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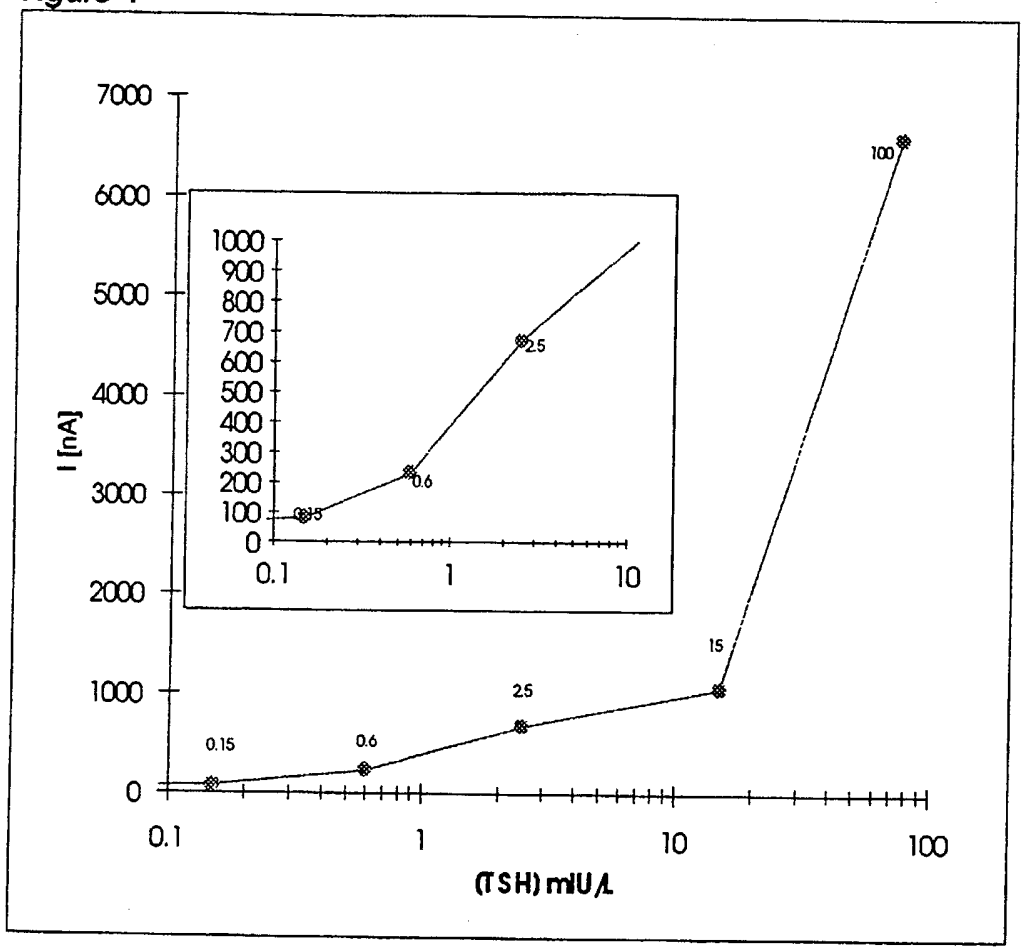
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0
50
100
150
200
250
300
350
400
T4ng/ml

Figure 4


7000
6000
5000
4000
3000
2000
1000
0
I [nA]
0.1
1
10
100
(TSH) mIU/L
0.15
0.6
2.5
15
100
1000
900
800
700
600
500
400
300
200
100
0
0.1
1
10
0.15
0.6
2.5

Flow - through Electrochemical Biosensor

Background of the Invention

The present invention relates to improvements in immunoassays and in particular to electrochemical immunoassay.
Immunoassay diagnostics are well established as the routine method of diagnosis for a wide range of substances in serum , plasma or other fluids and the principle of these assays can be extended to other fields such as environmental monitoring or DNA analysis.

The classical methods for immunoassay use either ;

(i) a capture antibody on a solid phase , such as a plastic microtitre plate, exposure to the biological sample to attach the antigen of interest , washing and then exposure to a second labelled antibody . The label on the antibody can be a radioactive tracer or enzyme for example . Further washing is followed by detection of the label (and hence the amount of antigen in the original sample) . This is known as a sandwich assay or two-site assay

or

(ii) a capture antibody on the solid phase followed by exposure to the biological sample containing antigen and an added amount of labelled antigen . Labelled and unlabelled antigen compete on the solid phase for the antibody sites . The amount of label revealed after washing is inversely proportional to the amount of true antigen in the biological sample . This is known as a competitive assay .

The revelation of label is achieved by various means . For enzyme labels the choices are chromogenic , fluorescent , luminescent or electrochemical detection . Chromogenic immunoassays , where the enzyme label produces a coloured product upon addition of a suitable substrate , are well established under the generic term ELISA , using spectrophotometric principles to detect the colour change in the substrate solution . They are relatively easy to perform but suffer from long incubation times and difficulty in achieving low limits of detection .

Luminescent and fluorescent products from a substrate solution can also be achieved via enzyme labelling and take the limits of detection lower by a factor of 10 for sandwich assays [1] when compared to chromogenic methods and significantly reduce the sample volumes required . Difficulties

arise in stabilising the substrates for commercial distribution and the expense of the luminescent \fluorescent detectors in the instrument costs .

Electrochemical immunoassay is considered to lie within the field of biosensors and essentially seeks to combine the detection of an electrochemically active species (which is the product of the enzyme - substrate reaction) by oxidation or reduction of the said species on an electrode in close proximity to the solid phase . There are many examples in the literature of such biosensors (see for examples [2]) but the majority have failed to achieve the required sensitivity and detection limits in any manner that could be used to fabricate a practical device for commercial use.

Another aspect of practical immunoassay is the implementation of assays on various solid phases of large surface areas in order to shorten the incubation times for the assay . Solid phases with large surface areas include polymer beads , magnetic particles and polymeric membranes .

Liquid flow technology in various forms has been applied in conjunction with a selection of solid phases to shorten assay times and enhance sensitivity. US- 5006-309-A describes a device with a membrane as a solid phase for immunoassay with optical detection . J6- 0188-839-A describes the use of a selective electrode which is projected into the stream of flow of a sample for detection .

EP- B1- 0033188 , EP- B1- 0060082 and EP- A1- 0469830 all describe the construction of porous electrodes that may be used to improve electrochemical detection in analytical systems such as HPLC. EP- B1- 0122009 is an electrode intended for the detection of some biological molecules which are electrochemically active provided that the samples are chromatographically filtered prior to examination. The electrodes described in these patents are intended to be used as a semi-permanent , re-usable detectors within chromatographic systems.
EP- A2 -0352138 and EP- A2- 0525723 both describe particular devices which incorporate membranes as solid phases and use the standard solid electrode formats normally employed in biosensors as electrochemical detectors. The devices are intended for use as disposables with a suitable instrumentation system.

Summary of the Invention

The first aspect of this invention is the use of a porous detection electrode to significantly augment the detection capabilities of electrochemical immunoassay by increasing the surface area of the electrode in contact with the electroactive species and enhancing the diffusion kinetics at the

electrode - species interface . The large surface areas provided by porous electrodes lead to extremely high oxidation and reduction efficiency of electroactive species under the proper flow and voltage conditions . The act of flowing the liquid through the electrode instantly replenishes the species for detection in the diffusion layer at the electrode -liquid interface as it is oxidised or reduced. This interface has been the limiting factor in many electrochemical immunosensors and other biosensors since the species as it is oxidised or reduced is depleted from the double layer and its replenishment has to be controlled , usually by stirring the solution above a solid detection electrode or flowing liquid over a solid detection electrode. The application of the electrode in this invention therefore covers improvements to two aspects of critical importance for electrodes to be used in electrochemical immunosensors or other biosensors , the need for high efficiency in oxidation and reduction (surface area) and the need to quickly replenish the double layer under controlled conditions (flow through measurement). The electrode can be any suitable porous conducting material such as pre-formed graphite sheets , fused carbon powders , metallic-based porous plugs or conducting inks printed on suitable porous bases.

The sensitivity of the electrode to a particular species can be manipulated by controlling flow speed and voltage at the electrode . Voltage control can be by the normal potentiostatic means to provide a current or charge reading in proportion to the amount of species in solution which is flowed through the electrode .Potentiostatic voltage control can be of any of the normal voltammetry or amperometry formats for electrochemical analysis but would preferably use the application of a dc. potential suitable for oxidation or reduction of the species and current at the electrode would be monitored by suitable instrumentation.

The electrochemically active species (also referred to as electroactive species) can be generated in the course of a sandwich or competitive or other immunoassay type by the use of any suitable enzyme - substrate pair which produces a readily oxidised or reduced species and the enzyme normally forms the assay label or part of the assay . The assay preferably uses the enzyme alkaline phosphatase and a phosphate substrate but could use any other suitable enzyme-substrate pair where the substrate generates an electrochemically detectable species on contact with the enzyme . For example the use of naphthyl phosphate in solution as the substrate in an alkaline phosphatase labelled immunoassay yields the electroactive species naphthol under appropriate buffer and substrate conditions . This can be readily oxidised under potentiostatic control on the porous electrode to yield one electron for every molecule of naphthol produced. Similarly aminophenyl phosphate can be used as a

substrate with alkaline phosphatase. Other examples of enzyme substrate pairs that could be employed are β-galactoxidase with p-Aminophenyl -β-D-galactoside to produce the electroactive species aminophenol , horseradish peroxidase with glucose to produce the electroactive species hydrogen peroxide and lactate dehydrogenase with lactate in the presence of NAD^+ to produce the electroactive species NADH .

The electrode is either incorporated in a suitable flow cell or device which allows the proper potentiostatic control of the electrode - liquid environment by the use of a three electrode system working (detection) electrode , counter and reference electrodes or in a device which requires only the presence of two electrodes , working and counter to properly function with an applied potential between the electrodes. The electrochemical detection can be carried out by means of peak current or total charge measurement and this can be calibrated against analyte concentration for each particular parameter subjected to electrochemical immunoassay in the system .

The second aspect of this invention is to incorporate the said porous electrode and the solid phase in a complete device to perform an immunoassay , in sequential flow steps or continuous flow , with high surface area and enhanced diffusion kinetics for both the antibody-antigen interactions on the solid phase and for the electrochemical detection phase , creating a device which is practicable and economic to fabricate requiring less sophisticated electronic control than either fluorescence or luminescence detection and providing very fast , sensitive diagnostic capabilities. The solid phase can be any suitable protein-binding material which demonstrates high surface area to volume ratios and good porosity such as polymer membranes , polymer beads or particle suspensions. Since the solid phase is preferably located close to the porous electrode and included in a complete immunoassay device the preferred solid phase is a polymer membrane .

A fuller description of possible configurations of the invention is now given and illustrated by the way of diagrams.

List of diagrams

Figure 1 shows a schematic cross section of a flow - through cell design incorporating a porous membrane to act as the solid phase for immunoassay and porous working and counter electrodes for electrochemical detection.

Figure 2. shows a schematic cross section of a flow through cell with wicking action .

Figure 3 is a diagram showing the results of the assay described in Example 3.
Figure 4 is a diagram showing the results of the assay described in Example 4.

Description of the device

Figure 1 illustrates a cross section through one possible configuration for a flow-through device or cell according to this invention for attachment to a pumped system for liquid flow control allowing the passage of the sample , reagent and wash solutions in a suitable sequence of steps through the cell to the final electrochemical detection step. The cell body 1 can be of any suitable pre-formed or machined chemically inert material but is preferably formed from a polymeric material and is illustrated in this case as being formed from two separable halves to allow the components of the device to be replaced as necessary. All reagents enter the cell through the circular aperture 2 and are pulled through in the direction of the arrow 3 by a pump attached below the cell with suitable tubing. The cell in this case is configured with a three electrode arrangement for full potentiostatic control where the porous electrode 5 is the working electrode , the porous electrode 6 is the counter electrode and a solid Ag\AgCl reference electrode 7 is exposed to contact with the reagent flow at the inner wall of the cell . The porous electrodes are fixed in position and sealed against the cell body by the aid of gaskets 10 ,11 and 12 and 'o' ring 13 as is the porous membrane 4 which provides the solid phase for the immunoassay reactions. The membrane 4 can be any suitable membrane for protein immobilisation but would preferably be made of one of the following materials ; nitrocellulose , polyvinylidene difluoride (PVDF), nylon or glass fibres. Pore diameter and thickness of the membrane can be tailored to the requirements of the particular immunoassay and required fluidic behaviour . The membrane 4 would be prepared according to the usual aspects of immunoassay known to those skilled in the art with capture antibody or antigen and inserted into the cell. The inner bore diameter , d, of the cell can be varied but preferred dimensions are in the range 2 -10mm to limit the sample volume required for proper detection of product. The thickness of the working electrode 5 should be dictated by the concentration range of the product to be detected to allow high oxidation or reduction efficiency and therefore high sensitivity or by the need to avoid rapid fouling of the electrode. Fouling describes the phenomenon whereby the oxidation or reduction of some electroactive species can create substances which are insoluble and which will gradually deposit on the electrode, blocking the surface and reducing its efficiency. Enhancing the available surface area of the electrode by selecting thicker porous electrodes increases the amount of time the electrode can be used before it must be replaced or the

concentration of species that can be detected before significant fouling occurs. Electrical connections are made through the cell to the electrodes and are connected externally to a potentiostat or other electroanalytical device with via contacts 7, 8 and 9.
The cell then forms the complete immunoassay environment for competitive or two-site electrochemical immunoassay as illustrated by Examples 3 and 4. The device conceptualised in Figure 1 could analyse plasma , serum or another fluid suitable for analysis by immunoassay.

The concept of the flow-electrochemical immunoassay device can also be implemented in a device adapted for wicking of solutions by capillary action in order that no pumping of fluids through the device need take place . The concept diagram of this type of device derived from the basic principles of the invention is illustrated by a cross section view though the device in Figure 2. Sample and reagents in the sequence required for a two-site or competitive immunoassay according to the parameter being tested enter the device at the aperture 14 and wick vertically through the solid phase membrane 15, spacer membrane 16a, porous working electrode 21, spacer membrane 16b , porous counter\reference electrode 22 and into absorbent material 19. Contacts are made to the working and counter electrodes via conducting materials 17 and 18 which can be suitably connected to a potentiostat or other electroanalytical device. The porous counter \reference electrode 22 is of a stable material but preferably of Ag\AgCl ink printed on a suitable porous support. The final reagent to be passed through the device is the substrate solution for the enzyme label which is instantly converted to electroactive product which is in turn detected by oxidation or reduction at the working electrode. The device is encapsulated in container 20 and is disposed of after one sample measurement. Plasma , serum or other fluids which can be analysed by immunoassay can be used on this device. In addition the device could be adapted for whole blood analysis by the addition of a blood \ plasma separation membrane at or adjacent to the test sample entrance point.
Devices can be realised from the conceptual drawings of Figures 1 and 2 by many different routes of fabrication and preparation. The porous electrode may be incorporated in the device directly as a pre-formed material or may be printed using a porous ink onto a suitable porous surface. The device may be constructed with a working, reference and counter electrode or simply as a two electrode system according to the demands of the analytical implementation of the device. To reduce generation of waste materials the device, of a type described in concept in Figure 1 could be constructed in two separable halves , one to contain the membrane or other antibody \ antigen capture phase and the other to contain the detection electrode system.

The electrode half of the device would be re-usable for a pre-determined number of tests and only that part of the device containing the solid phase would have to be removed and replaced for every new sample tested.

Examples

Example 1

A disc of porous graphite , diameter 5mm , thickness 0.35 mm, was cut from a sheet of commercially available material (Toray Industries, Japan). As a first test it was placed in a conventional planar electrode cell where the only liquid contact with the disc was at the upper surface of the graphite as in normal solid electrodes for biosensors. The cell for measurement contained an Ag \AgCl reference electrode and a titanium counter electrode and could be stirred to provide diffusion layer control in the conventional fashion . This provided a model for the measurement of electroactive species from electrochemical immunoassay on normal solid phase electrodes . The measurement of 10 μM naphthol solutions in diethanolamine DEA buffer (DEA 50mM, 100mM Na Cl , pH 9.6) with the working electrode poised at a voltage of +300mV against the reference electrode gave an average peak current response of 100 nA .
In a second test another disc electrode was cut from the graphite sheet and placed in a flow cell of the type illustrated in Figure 1. The contact diameter of the electrode with the liquid was 3 mm. Samples of naphthol, at various concentrations in DEA buffer ,were flowed through the cell at 300 μl min $^{-1}$ with the electrode poised at +300mV against the Ag\AgCl reference and the resulting current peaks for each 300μl sample volume were recorded . Peak currents of $\approx$ 100nA were recorded for naphthol concentrations of 100nM making the porous electrode 100 times more sensitive than the conventional solid electrode under these voltage and flow conditions. This sensitivity is achieved because the configuration of the cell allows the total oxidation or reduction of the electroactive species as it passes through the electrode at appropriate flow rate and applied potential. The peak current response can be increased by increasing the velocity of flow through the cell . In this manner it is possible to distinguish naphthol concentrations to at least 0.5 nM against background currents provided by the carrier solutions (biological buffers). The limits to this are set in practice by the fluidics of the system . In particular , velocities which are high enough to generate air bubbles within an open system should be avoided.

Example 2

A conventional ELISA sandwich assay was used to compare the standard chromogenic assay with an electrochemical flow detection system in an assay to determine levels of thyroid stimulating hormone (TSH) in serum standard solutions .

The assay was performed on a 96 well microtitre plate coated with a capture antibody for TSH . TSH standard solutions , 100 μl \well , of different concentrations were incubated for 1 hour on the plate , the wells were then washed and a solution of the anti - TSH secondary antibody, 100 μl \well , labelled with alkaline phosphatase (ALP), was incubated for 1 hour on the plate . The plate was again washed with buffer solution to remove any unbound secondary antibody .

Half of the wells in the microplate were then exposed to a 2.8 mM solution of a chromogenic substrate for ALP , p-nitro-phenyl phosphate, 300 μl\ well. The other half were exposed to one of the electrochemical substrates for ALP, naphthyl phosphate,at a concentration of 1mM , 300 μl\well . After an incubation of 20 mins at 37°C the solutions in the wells were either measured for colour development in a plate reader (chromogenic substrate) or extracted from the wells and measured amperometrically by the flow method described in Example 1 (electrochemical substrate) . The normal chromogenic assay typically could not measure reliably below levels of TSH of 1 mU l^{-1} while the electrochemical flow detection method continued to measure to 0.01 mU l^{-1} .

Example 3

A competitive immunoassay was prepared for thyroxin (T4) in a flow-through system . A T4-alkaline phosphatase (T4-ALP) conjugate was prepared from L-Thyroxin sodium salt and linked to alkaline phosphatase (Biozyme) using di-succinimidyl suberate (DSS).

The anti-T4 antibody was loaded at 2 µg ml^{-1} in carbonate buffer pH 9.6 on to 0.45 µm nitrocellulose membrane (Schliecher and Schuell) using an ELIFA membrane assay system (Pierce). The membrane was blocked with blocking solution.

Disks of membrane (6mm diameter) were cut from the prepared membrane and loaded into a flow cell of the type illustrated in Figure 1 . A new piece of membrane was used for every sample tested . For the antigen incubation step 1 μg ml^{-1} T4-ALP conjugate in Tris buffered saline , pH 8.6 was mixed with T4 standard solutions in a 1: 5 ratio to provide the competitive assay at a total sample volume of 200 μl . The assay steps in the cell which were carried out consecutively without interruption were as follows : (i) Passage of 1 ml of buffer solution through the cell , (ii) Passage

of the 200µl of sample \conjugate mix , (iii) Passage of 1 ml of buffer through cell as a wash step . (iv) Passage of 300 µl of substrate solution (1 mM naphthyl phosphate in buffer). The electroactive product of this incubation , naphthol , was detected directly at the electrode in the cell (Toray , 3mm diameter in contact with fluid) which was poised at +300 mV .v. the Ag\AgCl reference electrode. The results , in agreement with the form obtained from classical competitive assay are shown in Figure 3. The total assay time was 27 minutes at room temperature.

Example 4

A sandwich (or two- site) immunoassay for TSH was performed in continuous flow in the following manner :

Nitrocellulose membrane (Millipore) was loaded with a primary antibody against TSH at a concentration of 10 µg ml^{-1} in carbonate buffer using the ELIFA system (Pierce) . The membrane was blocked using blocking solution.

Discs of 6mm diameter were cut from the membrane and placed in the a flow cell of the configuration shown in Figure 1. Human serum standards containing TSH were passed through the membranes at a sample volume of 300µL , a new membrane and electrode were employed for each concentration . For the assay a sample was flowed through the system and immediately followed by a secondary antibody - alkaline phosphatase conjugate (Byk Gulden Italia) in solution and then 1.5 ml of wash solution . Finally, 300 µL of 1 mM naphthyl phosphate solution in DEA buffer was passed through the device. The cell was polarised only from the wash step onwards to reduce the risk of fouling the electrode with oxidation or reduction products from the serum sample.
Naphthol was detected immediately at the porous electrode by its oxidation current with the electrode poised at +300mV vs. an Ag\AgCl reference electrode . Total incubation time for the assay from sample addition to results was 29 minutes at room temperature Results are shown in Figure 4 and conform to the expected response from a sandwich or two-site immunoassay.

References

1. Portsmann, T. and Kiessig, S.T. " Enzyme Immunoassay Techniques " J. Immuno. Meth. , 150, pp 5-21, 1992.
2. Heineman , W.R. and Halsall, H.B. " Strategies for electrochemical immunoassay " Anal. Chem. , 57 ,2, pp 1321-1331 , 1985 .

Claims

1. A flow through solid phase immunoassay system or device with electrochemical detection capabilities , characterised in that the solid phase is in close connection with a porous working electrode and a suitable counter electrode is present in the system.

2. A flow through solid phase immunoassay system or device with electrochemical detection capabilities according to claim 1 , characterised in that the solid phase is at least one porous membrane in close connection with the porous working electrode.

3. A flow through solid phase immunoassay system or device with electrochemical detection capabilities according to claim 1 , characterised in that a flow control system is present.

4. A flow through solid phase immunoassay system or device with electrochemical detection capabilities according to claim 3, characterised in that the flow control system is a pump producing a pressure difference.

5. A flow through solid phase immunoassay system or device with electrochemical detection capabilities according to claim 3 , characterised in that the flow control system is represented by a wicking material.

6. A flow through solid phase immunoassay system or device with electrochemical detection capabilities according to claim 1 , characterised in that a reference electrode is also present.

7. A flow through solid phase immunoassay system or device with electrochemical detection capabilities according to claim 1 , characterised in that the porous working electrode is a graphite or carbon paper electrode or a printed electrode on porous material.

Patents Act 1977
Examiner's report to the Comptroller under Section 17
e Search report)

Application number
GB 9409449.7

Relevant Technical Fields

(i) UK Cl (Ed.M) G1N (NBMA, NBPE)

(ii) Int Cl (Ed.5) G01N 33/487, 33/543

Search Examiner
D MOBBS

Date of completion of Search
'29 JUNE 1994

Databases (see below)
(i) UK Patent Office collections of GB, EP, WO and US patent specifications.

(ii) ONLINE DATABASES: WPI

Documents considered relevant following a search in respect of Claims :-
1-7

Categories of documents

X: Document indicating lack of novelty or of inventive step.

Y: Document indicating lack of inventive step if combined with one or more other documents of the same category.

A: Document indicating technological background and/or state of the art.

P: Document published on or after the declared priority date but before the filing date of the present application.

E: Patent document published on or after, but with priority date earlier than, the filing date of the present application.

&: Member of the same patent family; corresponding document.

Category	Identity of document and relevant passages	Relevant to claim(s)
A	US 4927502 (UNIVERSITY OF TEXAS)	
X	WPI Abstract Accession No 90-168450/22 and JP 2110363 (TEIJIN) 23.04.90 (see abstract)	1, 2

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